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Presence of rhizobia nodulating common birdsfoot trefoil (*Lotus corniculatus* L.) in soils of Serbia

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A B S T R A C T

Common birdsfoot trefoil (*Lotus corniculatus* L.) is a perennial forage legume tolerant to adverse environmental conditions such as low soil pH, poor drainage, various insects and plant diseases. Owing to its ability to establish a symbiosis with nitrogen-fixing bacteria, rhizobia, thrives on soils of low fertility and is used in grass mixtures to enrich the soil with nitrogen. The aim of the work was to investigate the presence of rhizobia that nodulate birdsfoot trefoil in the soils of Serbia and to perform the isolation and evaluation of their nitrogen fixation potential. The presence and abundance of birdsfoot trefoil rhizobia in 28 soil samples from different localities and types of soil in Serbia, as well as different ways of use, was tested. From positive samples the rhizobial strains were isolated and their efficiency of nitrogen fixation was tested under controlled conditions. The presence of rhizobia that nodulate birdsfoot trefoil was recorded in 23 samples, while all 5 samples where the presence of rhizobia was not recorded are characterized by the absence of the host plant. The number of rhizobia in samples where there was no host plant was significantly less than in its presence. A total of 49 rhizobial strains were isolated, and in some samples of low abundance it was not possible to isolate strains. In controlled conditions during reinoculation, 80% of the strains were active, achieving 2.5 times greater dry above-ground mass than non-inoculated plants. The percentage of nitrogen in plants ranged from 1.6% in the case of inactive strains to 4.6% in the case of highly active strains. The results of the study indicate the necessity of inoculation, that is, the application of rhizobia on the lands where the common birdsfoot trefoil is sown for the first time due to their reduced number and activity.

Keywords: common birdsfoot trefoil, rhizobia, soils of Serbia

И З В О Д

Жути звездан (*Lotus corniculatus* L.) је вишегодишња крмна легуминоза, толерантна на неповољне услове животне средине као што су низак pH земљишта, лоша дренажа, разни инсекти и болести биљака. Захваљујући способности да успостави симбиозу са бактеријама које фиксирају азот, ризобијама, успева на земљиштима ниске плодности и користи се у травним смешама за обогаћивање земљишта азотом. Циљ рада је био да се испита присуство ризобија које нодулишу жути звездан у земљиштима Србије и да се изврши изолација и селекција сојева који су високо ефикасни у фиксацији азота као потенцијалних инокуланата. Испитано је присуство и бројност ризобија које нодулишу жути звездан у 28 узорака земљишта са територије Србије, узетих са различитих локалитета и типова земљишта, као и различитих начина коришћења, а затим су изоловани сојеви и тестирана ефикасност фиксације азота у контролисаним условима. У 23 узорка забележено је присуство ризобија која нодулишу жути звездан. Пет узорака земљишта код којих није забележено присуство ризобија карактерише одсуство биљке домаћина. Број ризобија у узорцима где није било биљке домаћина је знатно мањи него у њеном присуству. Изоловано је укупно 49 сојева, док из неких узорака са малом бројношћу није било могуће изоловати ризобијалне сојеве. У контролисаним условима током реинокулације, 80% сојева је било активно, постижући 2.5 пута већу суву надземну масу од неинокулисаних биљака. Проценат азота се кретао од 1.6% код неактивних сојева до 4.6% код високо активних сојева. Резултати студије указују на неопходност инокулације, односно примене ризобија на земљиштима на којима се жути звездан први пут сеје због њиховог смањеног броја и активности.

Кључне речи: жути звездан, ризобије, земљишта Србије.

1. Introduction

The common birdsfoot trefoil (*Lotus corniculatus* L.) is a valuable fodder legume with high nutritional value (www.feedipedia.org). Common birdsfoot trefoil is a perennial leguminous plant that regulary live for five to seven years, in the case of some wild forms even longer. Its tree is herbaceous, usually 40 cm high, and in favourable conditions up to 60-90 cm. The root is well developed, branched and penetrates into the soil up to depth of 1 - 1.5m (Stevanović, 2016). Common birdsfoot trefoil, like other leguminous plants, enters into a symbiotic association with nitrogen-fixing bacteria from the genus *Mesorhizobium* that form nodules on the roots and are also called root nodule bacteria – rhizobia. The rhizobial species *Mesorhizobium loti* is specific for birdsfoot trefoil, being its the most common simbiont (Lorite et al., 2010; 2018). The number and size of nodules depends on soil fertility, nutrition, and the age of the plant. The common birdsfoot trefoil with its strong roots can fix up to 200 kg ha^{-1} of nitrogen (Stevanović, 2016; Stević et al., 2017).

Average yield of birdsfoot trefoil in our country varied between 6 and 14 t ha^{-1} (Petrović et al., 2011). Production of forage depends on the age of crops, i.e. plant development, size of vegetation area and on the quantity of precipitation during the year (Petrović et al., 2011). Hay contains about 18% of crude protein. It has good digestibility, and does not cause flatulence in cattle. It is characterized by special values compared to other perennial legumes: it is not attacked by parasites and is resistant to leguminous diseases (Lorite et al., 2010).

The common birdsfoot trefoil thrives on less fertile and slightly more acidic soils. Unlike alfalfa, it tolerates acidic soils, and compared to red clover, it has a longer period of exploitation. It is part of almost all grass-leguminous mixtures. It is resistant to high temperatures and drought, as well as flooding. The common birdsfoot trefoil is often grown in mixtures with grasses and has a positive effect on their development, primarily due to the use of excess nitrogen created in the nitrogen fixation process. Bearing this in mind, inoculums that would contain rhizobia with appropriate plant growth promoting properties, (like auxin production, phosphate solubilization, etc.) would enable a more complete nutrition of both leguminous and non-leguminous plants that are grown with them, i.e. they would improve overall soil fertility (Knežević et al., 2021).

For the successful cultivation of legumes, including common birdsfoot trefoil, nodulation with effective strains of rhizobia that supply the plant and the soil with nitrogen is important (Vance, 1997). The density and activity of autochthonous rhizobia is diverse in different soils, so for the successful cultivation of legumes it is of great significance to know the presence of local natural populations of rhizobia in soils and their nitrogen fixation potential (Estrella et al., 2009; Sotelo et al., 2011). This enables the assessment of the need for artificial inoculation of legumes with highly active strains, as well as the isolation and selection of highly efficient strains of local areas (Delić et al., 2016).

In the world and in our country, many researches are carried out with the aim of forming a collection of nodule bacteria strains. Collections of strains of nodule bacteria contain autochthonous strains with different activities and serve as starting material for the selection of strains with different goals: highly efficient in nitrogen fixation, tolerant to different environmental factors (pH, heavy metals, drought, etc.) (Belechheb et al., 2021). This provides a source of potential strains originating from different habitats, which increases the possibility of selecting the highest quality strains for practical application in the production of quality inoculums. There are some researches regarding the influence of rhizobial inoculation on *Lotus corniculatus* plants in our country (Jarak et al., 2007; Stević et al., 2017; Knežević et al., 2022), however the studies of presence and number of native birdsfoot trefoil mesorhizobial strains in soils, are still limited.

The aim of the work was to investigate the presence and number of rhizobia that nodulate common birdsfoot trefoil in the soils of Serbia and to isolate and evaluate their nitrogen fixation potential.

2. Materials and methods

2.1. Soil sampling

Each soil sample represented one average sample, made of five individual samples, taken from both diagonals and midpoints for one plot. Sampling was carried out sterile, from a depth of 25 cm, without a surface layer of 0.5-1 cm. For rhizobium presence analysis, fresh soil was stored in plastic bags at 4°C.

2.2. Soil samples analyses

Soil pH was determined using a glass electrode pH meter in 1M KCl and in H_2O (soil: KCl or H_2O 1:2.5 ratio). Available P and K in soil were determined by the AL-method of Egner-Riehm (Egnér et al., 1960). Soil Ca and Mg were extracted with ammonium acetate and determined using a dual atomic adsorption spectrophotometer SensAA (Dandenong, Australia). Organic C and N in the soil were determined using an elementary CNS analyser, Vario model EL III (Hanau, Germany).

2.3. Determination of the presence and probable number of rhizobia in soil samples

Determination of the presence and probable number of rhizobia in soil samples was performed by the method of plant infection by dilutions of soil suspension in the controlled gnotobiotic conditions (Vincent, 1970). For plant cultivation Jensen's nitrogen-free medium was poured into the bacto-tubes, plugged with a cotton cap and autoclaved for 20 minutes at a temperature of 121°C. After autoclaving, the test tubes were bevelled to obtain the slight substrate slope. The seeds were surface sterilised by 96% alcohol for 0.5-1 minute with stirring, and after that with 0.2% $HgCl_2$ solution for 3-5 minutes. After the mercury solution was poured off, the seeds were washed 5-6 times with sterile distilled water.

2.3.1. Preparation of the dilution of the soil suspension

A dilution of the soil suspension of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were made with sterile physiological solution. Seven days old seedlings on agarised Jensen medium were inoculated with 0.5 ml of decimal dilutions of the tested soil.

2.3.2. Conditions for cultivation of birdsfoot trefoil

The plants were grown under controlled conditions in a light chamber, 16h day /8 h night regime. The average temperature during the experiment (measured daily at the same time) was

28°C-day/17°C-night. The presence of rhizobia nodulatin birdsfoot trefoil in the examined soils was determined visually, by the presence of nodules on the roots of plants inoculated with soil suspensions of different dilutions. The appearance of the first nodules was observed 3 weeks after inoculation, and the plants were grown for 6 weeks, after which the presence of nodules was recorded. The number of bacteria in 1 g of soil was calculated using the Mc Crady table (Vincent, 1970).

2.3.3. Isolation of strains

From the nodulated plants, root nodules were collected, surface sterilized by 96% ethanol for few seconds and then in 0.1% $HgCl_2$ solution (3–5 min), and rinsed by sterilized water 5-6 times (Vincent, 1970). Nodules were crushed in physiological solution and streaked on mannitol agar plates with Congo red. Plates were incubated for 5 days at 28 °C. Bacterial colonies selected according to the morphology were restreaked to obtain pure bacterial culture and Gram staining was performed for each isolate.

2.3.4. Strain nitrogen fixation potential

The nitrogen fixation efficiency of selected rhizobial isolates was evaluated in the test tubes under controlled conditions. Seeds of common birdsfoot trefoil were surface sterilized, and one seed was put in the test tube with 30 ml of Jensen agar (Vincent, 1970). Seeds were inoculated with 0.5 ml of bacterial suspension grown for 72h, and test tubes were kept in the light chamber. The two groups of control plants were used: plants without inoculation and without fertilization- control plants Ø, and plants fertilized with 0.05% KNO_3 solution and without inoculation – control plant with nitrogen NØ. The experiment was performed with 10 plants as replications. After six weeks of sowing the plants were sampled. The number of root nodules, plant height, root length and shoot dry weight (SDW) were recorded for each treatment. After sampling, plant material was dried in an oven at 70 °C, grounded, and the percentage of shoot N was determined by the CNS analyzer, Vario model EL III (Hanau, Germany).

3. Results and discussion

3.1. Rhizobial presence

The presence and number of rhizobia that nodulate birdsfoot trefoil in 28 soil samples from different locations, as well as different land use, with and without common birdsfoot trefoil presence (Table 1), were examined. The presence of rhizobia that nodulate birdsfoot trefoil was found in 24 out of 28 tested soil samples (85.7%, Figure 1). The rhizobia were present in different numbers in different types of soil, and the abundance ranged from 8 to 32.000 rhizobia g^{-1} soil (MPN), both in the presence and absence of the host plant. The presence of rhizobia was not recorded in 4 samples: 2 deriving from Mountin Avala and 2 from the south of Serbia, all from localities where there was no presence of birdsfoot trefoil. On the other hand, in 9 of 28 samples where there were no presence of common birdsfoot trefoil , the rhizobia were detected, even in very high number (sample 25, 27 and 28), indicating rhizobia existence regardless of the host plant presence.

Table 1.
Chemical composition of soil samples, presence and number of rhizobia nodulating common birdsfoot trefoil

Sample	Location	Soil use	*Lotus corniculatus* presence	pH	P_2O_5 mg/100g	K_2O mg/100g	Total N (%)	No of rhizobia per g of soil
1	Košutnjak	Meadow	-	5.70	23.48	43.60	0.34	8
2	Resnik	Meadow	-	4.30	2.69	14.86	0.30	0
3	Avala	Meadow	-	6.90	64.24	71.05	0.25	0
4	Jajinci	Meadow	-	5.90	41.13	26.23	0.29	8
5	Stara planina	Meadow	+	5.80	6.20	38.06	0.27	40
6	Stara planina	Meadow	+	6.00	44.35	156.40	0.75	40
7	Stara planina	Meadow	+	5.70	28.94	152.86	0.49	40
8	Stara planina	Meadow	+	7.60	3.29	15.23	0.17	40
9	Vučedolci	Meadow	+	4.50	15.75	128.64	0.75	400
10	Đorđinci	Meadow	+	5.30	2.47	33.31	0.24	6.000
11	Surdulica	Meadow	+	4.70	4.49	37.41	0.25	28.000
12	Vladičin Han	Meadow	+	5.60	6.06	54.74	0.32	4.000
13	Surdulica	Meadow	+	5.70	9.28	82.70	0.47	400
14	Pretina	Meadow	+	5.50	4.72	79.43	0.34	28.000
15	Đurdjevi stupovi	Meadow	+	nd	nd	nd	nd	50
16	Đurdjevi stupovi	Meadow	+	nd	nd	nd	nd	50
17	Đurdjevi stupovi	Meadow	+	nd	nd	nd	nd	8
18	Vladičin Han	Meadow	+	7.10	10.43	28.6	0.18	26
19	Lebane	Meadow	+	4.45	2.31	6	0.08	32.000
20	Leskovac	Arable land	-	5.10	7.89	12.6	0.15	600
21	Leskovac	Meadow	-	4.40	3.11	22.3	0.17	40
22	Vladičin Han	Garden	-	6.80	91.62	45	0.22	4.000
23	Vladičin Han	Wheat	-	5.10	9.09	11.88	0.09	0
24	Leskovac	Arable land	-	5.80	37.3	19	0.17	18
25	Lebane	Arable land	-	4.60	0.4	9.4	0.17	22.000
26	Leskovac	Meadow	-	6.90	37.6	45	0.27	0
27	Leskovac	Orchard	-	6.80	27.18	39.4	0.38	22.000
28	Vladičin Han	Wheat	-	6.80	9.88	34.6	0.23	28.000

Nd-not determined

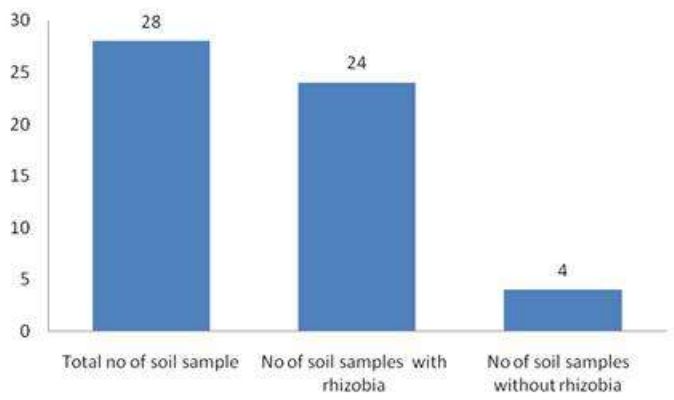

Figure 1. Number of soil samples with and without the presence of birdsfoot trefoil nodulating rhizobia

Previous experiments examined the nodulation of the birdsfoot trefoil in the soils of the Jablanica district and found nodulation in all the examined samples (12 locations) regardless of the type of soil, the land use system and the presence of the host plant (Vojinović et al., 1978). However, in the same study the efficiency of nitrogen fixation of autochthonous populations of birdsfoot trefoil rhizobia was weak, and thus indicated the need for artificial inoculation with effective strains in these soils as well.

Baraibar et al. (1999) recorded the presence of birdsfoot trefoil rhizobia in 10 examined soils without history of legume cultivation, and the strains isolated from all locations were effective in nitrogen fixation. In the same research, there were no correlation between *Rhizobium loti* densities and soil parameters such as pH, organic matter, organic nitrogen, phosphorous, Al^{3+} and clay content. This is in concordance with our results, where no correlation with the same parameters was observed (Table 2).

It used to be thought that the geographical distribution of rhizobia was related to the geographical distribution of specific host legumes (Vojinović and Petrović, 1961; 1968). However, the presence of strains in places without a history of growing specific legumes is attributed to natural dispersal mechanisms such as wind-borne dust or flowing water (Wang and Chen, 2004, Silva et al., 2005). It can be assumed that birdsfoot trefoil rhizobia were carried here by wind if the surrounding soil contains it, and the low number per gram of soil can be explained by the absence of a host plant on that surface. In general, the presence of *M. loti* is determined by the distribution area of the host plant and soil conditions to which *M. loti* is sensitive (pH, base saturation, presence of heavy metals). Several environmental variables such as pH, moisture, salinity, nitrogen, temperature, elevation and latitude have been reported to influence the biogeographic structure in rhizobium (Van Cauwenberghe and Honnay, 2015; Stefan et al., 2018; Zhang et al., 2018). Additionally, the composition of rhizobia may vary spatially, temporally and as a function of plant genotype (Zhang et al., 2011; Fan et al., 2017; Burghardt, 2020). Several phylogeographic studies have revealed spatial structure in the distribution of rhizobia (Silva et al., 2005; Cao et al., 2017; Stefan et al., 2018), while others have found no such patterns (Fierer and Jackson, 2006). Where found, spatial patterns were shown to be influenced by edaphic and climatic factors (Zahran, 1999; Bissett et al., 2010; Stefan et al., 2018).

Table 2.
Correlation between number of birdsfoot trefoil rhizobia and soil characteristics

	Land use	*Lotus* presence	pH	P	K	N	No of rhizobia
Land use	1						
Lotus presence	-0.510**	1					
pH	0.232	-0.061	1				
P	0.157	-0.358	0.487*	1			
K	-0.253	0.450*	0.063	0.295	1		
N	-0.210	0.377	-0.036	0.182	0.890**	1	
No of rhizobia	-0.066	0.109	-0.240	-0.181	0.171	0.268	1

* Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level.

3.2. Nitrogen fixation efficiency of strains.

From nodules formed from all soil samples, a total of 29 isolated strains were identified as rhizobia on the basis of the morphological and physiological properties and chosen for further testing. Out of 29 strains, after reinoculation of plants, 22 strains formed nodules on birdsfoot trefoil (about 75% of the isolated strains) and showed positive effects on plant growth.

Indicators of strain nitrogen fixing activity can be visual: plant height, general appearance and development of plant, number of leaves, number and appearance of nodules. Plants inoculated with active strains are very green, tall, and some have branches. Most of the active strains form large, pink nodules in small numbers on the roots, which is an indicator of their nitrogen fixation efficiency.

Table 3.
Average values of investigated parameters in inoculated and noninoculated plants of birdsfoot trefoil

	Minimum	Maximum	Mean	Std. Error	Std. Deviation	Variance	Ø	NØ
Plant height (cm)	13.36	20.00	16.55	0.41	1.91	3.66	6.29	19.84
Root length (cm)	10.60	18.50	14.21	0.36	1.69	2.85	14.25	9.90
Number of nodules per plant	4.44	11.67	8.00	0.48	2.27	5.14	0	0
Shoot dry weight (mg/plant)	9.06	31.52	17.04	1.19	5.43	29.51	4.97	22.2
N percentage	2.86	4.49	4.08	0.09	0.42	0.18	1.16	5.08
Total N in shoot (mg/plant)	0.26	1.33	0.71	0.05	0.24	0.06	0.05	1.12
Fixed N in shoot (mg/plant)	0.21	1.28	0.66	0.05	0.24	0.06	0	0

Ø-Control noninoculated plants, NØ-Control noninoculated plants with mineral nitrogen

The height of inoculated plants, shoot dry weight (SDW-shoot dry weight) as well as percentage and total nitrogen content in SDW are used as indicators of nitrogen fixation efficiency. In addition to these parameters, the number of nodules per plant and the dry mass of the roots are also used, but it is considered that SDW is the best indicator of nitrogen fixation efficiency (Hungria and Bohrer, 2000; Hefny et al., 2001).

The mean value of the height of plants inoculated with tested bacterial isolates ranges from 13.36 to 20.00 cm (Table 3). The average number of nodules ranged from 4.44 to 11.67 in plants inoculated with rhizobia isolates, and mostly occurred 14 days after inoculation (Table 3). The large nodules of pink color, which are most often found on the root neck or main root, are active, while the small white ones are created by the inactive strain of rhizobia. The average value of the root length of plants inoculated with different rhizobia isolates ranges from 10.06 to 18.50 cm. (Table 3). The average SDW value of plants inoculated with different rhizobia isolates ranges from 9.06 to 31.52 mg/plant. The lowest value of SDW was obtained in control non-inoculated plants, 4.97 mg/plant (Ø-control without nitrogen). Some strains showed SDW values higher than control plants with mineral nitrogen, which was 22 mg/plant (NØ).

Based on Pochon's criteria (Pochons, 1954), strains are divided into active and inactive: strains are active in nitrogen fixation if they achieve a weight of above-ground dry mass (SDW) 2.5 times higher than the control without inoculation and addition of mineral nitrogen (Ø). The 82% of the isolates achieved a weight of SDW 2.5 higher than the control without nitrogen addition, and based on the criteria of Pochon, they are considered active strains.

The effectiveness of strains ie. symbiotic efficiency can be expressed through the ratio of SDW achieved in control (NØ) and inoculated plants (Gibson, 1987). Strains can be considered highly efficient if they achieve SDW values higher than 70% of the SDW of control plants with the addition of mineral nitrogen (NØ), which was achieved by 30% of the strains in our research. This research showed the presence of a large number of active isolates (80% of isolates), where 30% of the strains were highly efficient with SE values above 70%. However, the isolates completely inactive in symbiotic nitrogen fixation were also found in Serbian soils.

The percentage of nitrogen in inoculated plants ranged from 2.86 to 4.49%. The lowest percentage of nitrogen was in the control non-inoculated plants (Ø), 1.16% nitrogen, and the highest in the control plants supplemented with mineral nitrogen, 5.08% (NØ).

The presence of effective rhizobial strains did not depend on the presence of the host plant, since active strains were isolated both from alfalfa fields and from arable land, which is in agreement with previous studies (Vukmir, 1993). From some samples with a small number of rhizobia and without the presence of a host plant, conventional microbiological methods were used to isolate strains that were unable to nodulate birdsfoot trefoil when reinoculated, such as samples 1, 4, 16, 13 (Table 1). However, from the samples with low numbers and without the presence of the host plant, efficient strains in nodulation and nitrogen fixation were also isolated, samples 24, 18, 21 (Table 1), therefore no precise conclusion can be made about the connection between the absence of rhizobia and the nodulation of the host plant without a more detailed study with a larger by the number of soil samples.

Total nitrogen content was calculated from SDW mg/plant and total nitrogen percentage. Some inoculated plants that have the highest N percentages are not at the same time the plants with the highest total nitrogen content (Table 3). According to the data, the total nitrogen content was not in correlation with the percentage of nitrogen in plants (r=0.369). Fixed nitrogen was calculated by subtracting the value of total nitrogen obtained in the non-inoculated control Ø, from the total nitrogen in the inoculated plants. Fixed nitrogen ranged from 0.21 mg/plant to 1.28 mg/plant (Table 3).

Table 4.
Correlation between plant parameters examined

	Plant height	Root length	No of nodules	SDW	N %	Total N
Root length	0.253					
No of nodules	-0.120	0.204				
SDW	0.200	0.435*	0.573**			
N %	-0.054	0.122	0.077	0.176		
Total N	0.172	0.438	0.539*	0.978**	0.369	
Fixed N	0.172	0.438	0.539*	0.978**	0.369	1.000**

* Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level.

Correlations between individual parameters were examined (Table 4), and significant correlation was recorded between SDW of plants and root length (r=0.435**), SDW and number of nodules (r=0.573**), SDW and total and fixed nitrogen (r=0.978**). Number of nodules correlated positively with total and fixed nitrogen (r=0.539*). There was no correlation between SDW and N%, as well as the number of nodules and N%. These results are in agreement with some earlier studies where highly statistically significant correlations were observed between shoot dry weight and total N accumulated in shoots and in plants (correlation coefficients r=0.911 and r=0.915, respectively) (Hungria and Bohrer, 2000). Therefore, evaluation of shoot dry weight is a good indicator for the selection of highly efficient strains in nitrogen fixation.

4. Conclusions

The presence of rhizobia that nodulate birdsfoot trefoil was recorded in samples where the the host plant was present as well as in the samples characterized by the absence of the host plant. The number of rhizobia in samples where there was no host plant is significantly less than in its presence. The results of the study indicate the necessity of rhizobial inoculation, especially in the case of the lands where the common birdsfoot trefoil is sown for the first time, in order to circumvent the reduced number and activity of the naturaly exsisted rhizobia.

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Declaration of competing interests

Authors declare no conflict of interest.

References

Baraibar, A., Frioni, L., Guedes, E.M., Ljunggren, H. (1999). Symbiotic effectiveness and ecological characterization of indigenous *Rhizobium loti* populations in Uruguay. *Pesquisa Agropecuaria Brasileira*, 4, 1011-1017.

Belechheb, T., Bouhnik, O., Bakkali, M., Zerroukm, M.H., Laglaoui, A., Missbah El Idrissi, M., Arakrak. A. (2021). *Ensifer meliloti* sv. lancerottense nodulates *Lotus creticus* in alkaline soils of Northern Morocco. *Rhizosphere*, 18. 100339. doi: 10.1016/j.rhisph.2021.100339

Bissett, A., Richardson, A.E., Baker, G., Wakelin, S., Thrall, P.H. (2010). Life history determines biogeographical patterns of soil bacterial communities over multiple spatial scales. *Molecula Ecology*, 19(19), 4315–27.

Burghardt, L.T. (2020). Evolving together. evolving apart: measuring the fitness of rhizobial bacteria in and out of symbiosis with leguminous plants. *New Phytologist*, 228, 28–34.

Cao, Y., Wang, E., Tong, W. (2017). Population structure of Rhizobium etli-like strains nodulated with Phaseolus vulgaris in two ecoregions of China. *Soil Biology and Biochemistry*, 112, 14–23.

Gibson, AH. 1987. Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. Symbiotic Nitrogen Fixation Technology, In: Elkan, G.H. (Eds), Marcel Dekker Inc., New York, pp. 321–363.

Delić, D., Stajković-Srbinović, O., Knežević-Vukčević, J. (2016). Alfalfa (*Medicago sativa* L.) and *Sinorhizobium meliloti*: prospects of using rhizobial inoculants in Serbia. *Botanica Serbica*, 40, 13-19.

Fan, K., Cardona, C., Li, Y., Shi, Y., Xiang, X., Shen, C., Wang, H., Gilbert, J.A., Chu, H. (2017). Rhizosphere-associated bacterial network structure and spatial distribution differ significantly from bulk soil in wheat crop fields. *Soil Biology and Biochemistry*, 113, 275–84.

www.feedipedia.org

Hefny, M., Dolinski, R., Malek, W. (2001). Variation in symbiotic characters of alfalfa cultivars inoculated with *Sinorhizobium meliloti* strains. *Biology and Fertility of Soils*, 33, 435-437.

Hungria, M., Bohrer, T.R.J. (2000). Variability of nodulation and dinitrogen fixation capacity among soybean cultivars. *Biology and Fertility of Soils*, 31, 45-52.

Estrella, M.J., Munoz, S., Soto, M.J., Ruiz, O., Sanjuán, J. (2009). Genetic diversity and host range of rhizobia nodulating *Lotus tenuis* in typical soils of the Salado River Basin (Argentina). *Applied Environmental Microbiology*, 75, 1088–1098.

Egnér, H., Riehm, H., Domingo, W.R. (1960). Untersuchungen über die chemische Bodenanalyse als Grundlage für die Beurteilung des Nährstoffzustandes der Böden. II. Chemische Extraktionsmethoden zur Phosphor- und Kaliumbestimmung. *Kungliga Lantbrukshögskolans Annaler*, 26, 199-215.

Fierer, N., Jackson, R.B. (2006) The diversity and biogeography of soil bacterial communities. PNAS 103 (3), 626–631.

Jarak, M., Đurić, S., Jević, M. (2007). The effect of single and combined inoculation on the emergence and early growth of bird's-foot trefoil (*Lotus corniculatus*). *Letopis naučnih radova Poljoprivrednog fakulteta*, 2, 41–46.

Knežević, M., Berić, T., Buntić, A., Delić, D., Nikolić, I., Stanković, S., Stajković-Srbinović, O. (2021). Potential of root nodule nonrhizobial endophytic bacteria for growth promotion of *Lotus corniculatus* L. and *Dactylis glomerata* L. *Journal of Applied Microbiology*, 31, 2929-2940.

Knežević, M., Berić, T., Buntića, A., Jovkovića, M., Avdović, M., Stanković, S., Delić, D., Stajković-Srbinović, O. (2022). Native *Mesorhizobium* strains improve yield and nutrient composition of the common bird's-foot trefoil grown in an acid soil. *Rhizosphere*, 21, 100487.

Lorite, M.J., Muñoz, S., Olivares, J., Soto, M.J, Sanjuán, J. (2010). Characterization of strains unlike *Mesorhizobium loti* that nodulate *Lotus* spp. in saline soils of Granada. Spain. *Applied and Environmental Microbiology*, 76, 4019–4026.

Lorite, M.J., Estrella, M.J., Escaray, F.J., Sannazzaro, A., Videira e Castro, I.M., Monza, J., Sanjuán, J., León-Barrios, M. (2018). The Rhizobia-Lotus Symbioses: Deeply Specific and Widely Diverse. *Frontiers in Microbiology*, 9, 2055. doi: 10.3389/fmicb.2018.02055

Pochon, J. (1954). Manuel technique D'Analyse Microbiologique du sol. Masson et Cie, Paris.

Petrović, S., Vučković, S., Simić, A. (2011). Stand density effects on birdsfoot trefoil herbage yield grown for combined usage. *Biotechnology in Animal Husbandry*, 27, 1523-1530.

Silva, C., Vinuesa, P., Eguiarte, L.E., Souza, V., Martnez-Romero, E. (2005). Evolutionary genetics and biogeographic structure of *Rhizobium gallicum* sensu lato. a widely distributed bacterial symbiont of diverse legumes. *Molecular Ecology*, 14, 4033–4050.

Sotelo, M., Irusarri, P., Lorite, M., Casaretto, E., Rebufo, M., Sajuan, J., Monza, J. (2011). Diversity of rhizobia nodulating *Lotus corniculatus* grown in nothern and southern regions of Uruguay. *Applied Soil Ecology*, 49, 197-207.

Stefan, A., Van Cauwenberghe, J., Rosu, C.M., Stedel, C., Labrou, N.E., Flemetakis, E., Efrose, R.C. (2018). Genetic diversity and structure of *Rhizobium leguminosarum* populations associated with clover plants are influenced by local environmental variables. *Systematic and Applied Microbiology*, 41, 251-259.

Stevanović, P.R. (2016). Uticaj načina setve. količine semena i djubrenja na prinos i kvalitet semena žutog zvezdana (*Lotus corniculatus* L.) na zemljištu pseudoglej. Doktorska disertacija. Poljoprivredni fakultet. Univerzitet u Beogradu.

Stevović, V., Lazarević, Đ., Tomić, D., Delić, D., Đurović, D. (2017). Pre-sowing seed inoculation in the birdsfoot trefoil seed production. *Agrofor international Journal*, 2, 41-47.

Vance, C.P. (1997). Enhanced agricultural sustainability through biological nitrogen fixation. In: Legocki A, Bothe H & Pühler A (eds.), Biological fixation of nitrogen for ecology and sustainable agriculture, pp. 179-186, Springer-Verlag, Berlin.

Van Cauwenberghe, J., Michiels, J., Honnay, O. (2015). Effects of local environmental variables and geographical location on the genetic diversity and composition of *Rhizobium leguminosarum* nodulating *Vicia cracca* populations. *Soil Biology and Biochemistry*, 90, 71–79.

Vincent, J.M. (1970). A Manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific, Oxford.

Vojinović, Ž., Petrović, V. (1961). Rasprostranjenost nekih važnijih kvržičnih bakterija u zemljištima N.R. Srbije. *Arhiv za poljoprivredne nauke*.

Vojinović, Ž., Petrović, V. (1968). Prilog proučavanju rasprostranjenosti kvržičnih bakterija važnijih leguminoza u raznim zemljištima SR Srbije. *Zbornik instituta za proučavanje zemljišta,* 1, 127-145.

Vojinović, Ž., Petrović, V., Miličić, B. (1978). Prisustvo kvržičnih bakterija u zemljištima sliva reke Jablanice i reagovanje lucerke na inokulaciju. *Mikrobiologija*, 15, 95-104.

Vukmir, D.I. (1993). Odredjivanje prisustva i aktivnosti bakterije *Rhizobium meliloti* u različitim tipovima zemljišta kruševačkog regiona. Magistarski rad. Univerzitet u Beogradu, Beograd.

Wang, E., Chen, W.X. (2004). Estimation of biotic and abiotic factors that affect migration of rhizobia. Biological Resources and Migration. In. Werner D (ed). pp. 167–171, Springer Verlag. Berlin.

Zhang, B., Du, N., Li, Y., Shi, P., Wei, G. (2018). Distinct biogeographic patterns of rhizobia and non-rhizobial endophytes associated with soybean nodules across China. *Science of the Total Environment*, 643, 569–78.

Zhang, Y.M., Li, Y., Chen, W.F., Wang, E.T., Tian, C.F.,Li, Q.Q., Zhang, Y.Z., Sui, X.H.,Chen, W.X. (2011). Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain. *Applied and Environmental Microbiology*, 77, 6331–42.